Journal of Chromatography, 368 (1986) 85–93
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 856

FLUORESCENCE DETECTED CIRCULAR DICHROISM AS A DETECTION PRINCIPLE IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ROBERT E. SYNOVEC and EDWARD S. YEUNG*
Ames Laboratory-USDOE and Department of Chemistry, Iowa State University, Ames, IA 50011 (U.S.A.)
(Received April 25th, 1986)

SUMMARY

A highly sensitive and selective laser-based detector for high-performance liquid chromatography is presented. The detector combines the selectivity of fluorescence and the selectivity of optical activity to provide specificity. A HeCd laser operating at 325 nm with about 8 mW power is used with electro-optic modulation at 150 kHz. As a highly sensitive optical activity detector, the detection limit is 170 pg injected riboflavin using conventional liquid chromatography.

INTRODUCTION

High-performance liquid chromatography (HPLC) is extensively applied in the analysis of complex, often biologically related, samples. If the separation process is not adequate in providing the extent of selectivity required in an analysis, an appropriate detector must be chosen[1]. Optical activity detection (OAD) is a method that can provide excellent selectivity in HPLC analyses. OAD-HPLC procedures using polarimetry as the detection principle have been reported for the analysis of carbohydrates in urine[2] and cholesterol in human serum[3]. Optically active species in the samples are detected, exclusively, while optically inactive species pass through the detector unnoticed. Even greater selectivity in HPLC detection may be useful as complexity increases for the samples.

Riboflavin (vitamin B_2) is an important chemical species that exhibits both strong fluorescence and optical activity. Various analytical procedures for its analysis have been reported[4–6]. Using a commercially available fluorescence detector, with a conventional light source, and conventional HPLC, these procedures typically produced detectabilities of low nanogram levels of injected riboflavin. The need for a more sensitive procedure for riboflavin was suggested[4]. A laser fluorometric study of riboflavin provided data that imply that picogram detectabilities are possible for conventional HPLC[7]. Also, it is common for riboflavin to exist in a sample matrix that is quite complex, and traditional fluorescence detection, while selective to some extent, may not be adequate for the analysis. Circular dichroism (CD) spectroscopy of riboflavin and its analogues suggests that CD may provide an added dimension of selectivity when coupled with fluorescence[8].

0021-9673/86/$03.50

The use of fluorescence detected circular dichroism (FDCD) in the analysis of biologically significant systems in static solutions[9] provided invaluable structural information. This application suggests the development of an HPLC detection principle based upon FDCD. Similarly, the report of a transmission mode (TM) CD detector for HPLC[10] supports the claims of improved selectivity and ample sensitivity that an FDCD detector for HPLC should provide.

In this paper, a laser-based FDCD–HPLC system has been developed, and tested with a reversed-phase chromatographic separation of a mixture containing riboflavin. Some of the problems associated with CD detection in dynamic (flowing) systems, as compared to static systems, are discussed. Also, experimental and technical considerations concerning FDCD–HPLC and TM CD–HPLC[10] will be presented. FDCD–HPLC is found to provide ample sensitivity (170 pg limit of detection for riboflavin), while also providing the detection selectivity of optically active fluorophores in the presence of optically inactive fluorophores.

THEORY

CD is defined as the difference in absorbance of left circularly polarized light (LCPL) and right circularly polarized light (RCPL). Since CD is dependent upon absorbance, the CD parameter, $\Delta\varepsilon$, is related to the molar absorptivities for LCPL and RCPL by

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R \tag{1}$$

where L indicates LCPL and R indicates RCPL. In order to use eqn. 1 for FDCD, an expression relating the fluorescence, I_F, detected at 90° from an incident light source I_0, for small absorbance, is useful.

$$I_F = 2.303\, f(\theta)g(\lambda)\varphi_F\varepsilon bCI_0 \tag{2}$$

where $f(\theta)$ is a geometric collection efficiency factor, $g(\lambda)$ is a signal conversion factor depending upon wavelength, φ_F is a factor accounting for quantum efficiency of fluorescence, ε is the average molar absorptivity, b is the observed pathlength, and C is the concentration of a fluorophore.

Ideally, the light source incident upon a FDCD detection cell is modulated at a given frequency, so that one half-cycle is entirely RCPL and the second half-cycle is entirely LCPL. In practice, this is not the case, but rather

$$\Delta I_0 = I_{0,L} - I_{0,R} \tag{3}$$

with

$$I_0 = (I_{0,L} + I_{0,R})/2 \tag{4}$$

It is also useful to note that

$$\varepsilon = (\varepsilon_L + \varepsilon_R)/2 \tag{5}$$

for optically active species. Thus, for a lock-in detection system, the signal detected in the FDCD measurement, ΔI_F, will be related to the fluorescence intensity detected on each half-cycle of modulation

$$\Delta I_F = I_{F,R} - I_{F,L} \tag{6}$$

where $I_{F,R}$ is the fluorescence intensity due to RCPL, and $I_{F,L}$ is the fluorescence intensity due to LCPL. Taking eqns. 1 and 2 and assuming ΔI_0 is equal to zero, the FDCD signal is expressed by

$$\Delta I_F = -2.303\ f(\theta)g(\lambda)\varphi_F \Delta\varepsilon bCI_0 \tag{7}$$

The negative sign indicates that the FDCD signal is opposite in magnitude as compared to the TMCD signal[10], according to the definition of $\Delta\varepsilon$ in eqn. 1. Eqn. 7 is consistent with what has previously been reported[11] for the FDCD measurement in static systems. Eqn. 7 is an ideal expression, since ΔI_0 cannot be neglected in practice due to imperfections in the electro-optic modulation process and due to difficulties in balancing birefringent and reflection phenomena in the detection cell[12]. The ΔI_0 effect on ΔI_F can be included on an additive basis, since very small absorbances are measured in a chromatography context. For an optically active fluorophore, $\Delta I_{F,OA}$ is given by

$$\Delta I_{F,OA} = 2.303\ f(\theta)g(\lambda)\varphi_F bC[-\Delta\varepsilon I_0 \pm \varepsilon\Delta I_0] \tag{8}$$

where the first term in the brackets is for the CD effect, and the $\pm$ sign on the second term indicates that ΔI_0 can arbitrarily be positive or negative relative to the first term[10]. For an optically inactive fluorophore, an experimentally measured signal, $\Delta I_{F,OI}$, due entirely to ΔI_0 may be observed

$$\Delta I_{F,OI} = 2.303\ f(\theta)g(\lambda)\varphi_F bC[\pm\varepsilon\Delta I_0] \tag{9}$$

Note that for a given measurement, the $\pm$ sign in eqns. 8 and 9 will be the same.

It is possible to measure both the fluorescence and the FDCD signal[13] for the same chromatographic system. For an optically active fluorophore, using eqns. 2 and 8, the ratio of $\Delta I_{F,OA}$ and I_F is given by

$$\frac{\Delta I_{F,OA}}{I_F} = -\frac{\Delta\varepsilon}{\varepsilon} \pm \frac{\Delta I_0}{I_0} \tag{10}$$

where ε is defined as in eqn. 5 and I_0 is defined by eqn. 4. For an optically inactive fluorophore, using eqns. 2 and 9, the ratio of $\Delta I_{F,OI}$ and I_F yields

$$\frac{\Delta I_{F,OI}}{I_F} = \pm\frac{\Delta I_0}{I_0} \tag{11}$$

Again, the $\pm$ sign in eqns. 10 and 11 will be the same. Thus, if the effect due to ΔI_0 cannot be neglected, then the use of an optically inactive fluorophore, via eqn. 11,

provides a means to subtract off this unwanted contribution in eqn. 10 for optically active fluorophores. This procedure will be confirmed in this work, by utilizing it in HPLC coupled with FDCD detection.

EXPERIMENTAL

Detection system

The detection system is laser-based, modulated by using polarization techniques, and can be seen in Fig. 1. The 325-nm light from the HeCd laser (Liconix, Sunnyvale, CA, U.S.A. Model 4240NB) at about 8 mW power is sent through a 50-cm focal length quartz lens, and on through an electro-optic modulator (Lasermetric, Teaneck, NJ, U.S.A., Model 3030). After exiting the modulator, the light enters the chromatographic detection cell. The detection cell is approximately at the focal point of the quartz lens. Any fluorescence originating in the detection cell is collected 90° from the incident laser beam direction. This fluorescence passes through two filters (Corning Glass, Corning, NY, U.S.A., 4-65 and 0-52) before detection with a photomultiplier tube (RCA, Harrison, NJ, U.S.A., Type 1P28), which is biased at 1000 V. The detected signal is sent through an AC amplifier with a gain of 100, and on to a lock-in amplifier (Princeton Applied Research, Princeton, NJ, U.S.A., Model 5202), for phase-sensitive detection synchronized to the modulation frequency applied to the electro-optic modulation device. The output from the lock-in amplifier is sent to a strip chart recorder operating at 1 V full scale.

The polarization of the laser beam initially is vertical to the plane of the optical table. The electro-optic modulator (*i.e.* Pockels cell) acts upon the polarized laser light, in conjunction with a modulation driver (Conoptics, Danbury, CT, U.S.A., Model 25), which in turn is synchronized with the lock-in amplifier via a wave gen-

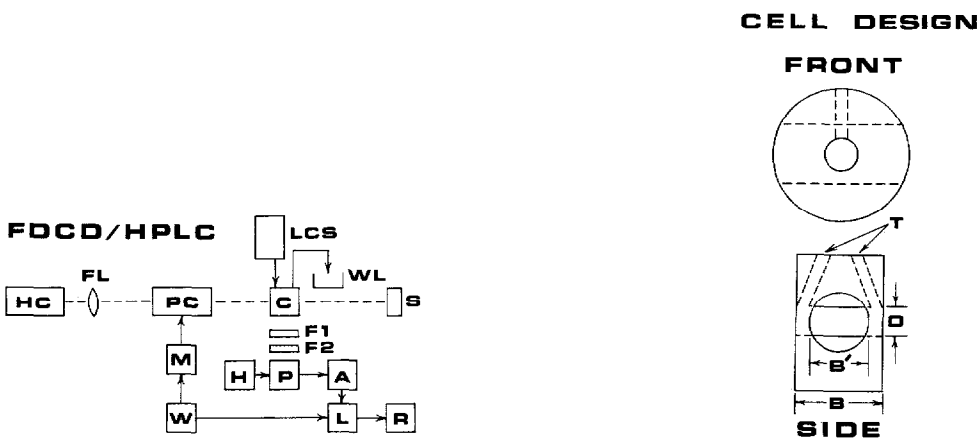

Fig. 1. FDCD–HPLC system. HC = HeCd laser, 8 mW; FL = 50-cm focal length quartz lens; PC = electro-optic modulator (Pockels cell); M = modulation driver; W = waveform generator; LCS = liquid chromatography system; WL = waste liquid; C = detection cell (detailed in Fig. 2); S = beam stop; F1 = 4-65 Corning filter; F2 = 0-52 Corning filter; P = photomultiplier tube; H = high voltage power supply; A = AC amplifier; L = lock-in amplifier; R = chart recorder.

Fig. 2. Chromatographic detection cell. B = 1.8 cm total pathlength; B′ = 1.2 cm observed pathlength; D = 1.0 mm I.D. quartz tubing; T = chromatography tubing, inlet and outlet.

erator (Wavetek, San Diego, CA, U.S.A., Model 162). The system is operated at 150 kHz with a square wave from the wave generator. It is possible to obtain CPL directly from the Pockels cell without using a Fresnel rhomb prism. This is in contrast to previous work[10]. By proper adjustment of the modulation driver bias voltage and input waveform peak-to-peak voltage, both right and then left CPL can be produced[14]. Yet, the production of CPL from the Pockels cell must be tested with a Fresnel rhomb, a polarizing prism, and a photodiode detector. Once CPL is obtained with the Pockels cell, the detection system for FDCD is simpler and just as effective as that reported for TMCD[10].

Chromatography system

The eluent consisted of 20% acetonitrile (Burdick and Jackson, Muskegon, MI, U.S.A., HPLC grade) and 80%, by volume, water, initially deionized and purified with a commercial system (Millipore, Bedford, MA, U.S.A., Milli-Q System). The liquid chromatography system consisted of a syringe pump (ISCO, Lincoln, NB, U.S.A., Model 314), an injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7410) with a 1-μl injection loop, and a 15 cm $\times$ 2.1 mm I.D. 5-μm C_{18} chromatography column (Alltech Assoc., Deerfield, IL, U.S.A.), which was connected to a detection cell (made in-house, shown in Fig. 2), having an observation pathlength of 1.2 cm and a cell volume of 14 μl. A flow-rate of 200 μl per min was used. The detection cell is positioned, with care, using a combination of translational and rotational stages (Aerotech, Pittsburgh, PA, U.S.A., Model ATS-301 and ATS-301R).

Samples studied

Both the optically active fluorophore (−)-riboflavin and the optically inactive fluorophore 4-methylumbelliferone were commercially available, reagent grade chemicals. They were found to be sufficiently pure for this work. Riboflavin and 4-methylumbelliferone both exhibit large absorptivities at 325 nm. Log ε values are 3.3 and 4.1, respectively, for these compounds. Also, they both exhibit intense fluorescence. The maximum fluorescence for riboflavin is near 535 nm, while that of 4-methylumbelliferone is below 500 nm. The filters were chosen to pass the fluorescence of riboflavin, but a significant amount of 4-methylumbelliferone fluorescence was also allowed to pass. From the literature[8], the optically active (−)-riboflavin has $\Delta\varepsilon$ values ranging from +1.5 to +2.5 l cm^{-1} mol^{-1} in solvent systems such as that used in this study. Concentrations injected into the HPLC system were $1.05 \cdot 10^{-5}$ *M* riboflavin and $4.25 \cdot 10^{-6}$ *M* 4-methylumbelliferone. Riboflavin has a molecular weight of 376 g/mole.

Fluorescence and FDCD measurements

A fluorescence chromatogram was obtained by inserting a Glan-Thompson polarizing prism (Karl Lambrecht, Chicago, IL, U.S.A., Model MGLA-SN-8) after the Pockels cell but before the detection cell. The modulation system was adjusted to form a polarization modulated laser beam that is "on" for one half-cycle, and "off" for the other half-cycle. Lock-in detection provides a fluorescence signal proportional to that described by eqn. 2. A series of at least three fluorescence chromatograms were obtained until good precision was substantiated by the reproducibility.

A FDCD chromatogram was obtained by removing the polarization prism and adjusting the modulation system to produce, alternately, RCPL and LCPL as a function of the modulation frequency. Lock-in detection provides a FDCD signal proportional to that described by eqns. 8 and 9. This is done only after the ratio $\Delta I_0/I_0$ has been experimentally minimized to reduce this extraneous contribution to the signal (see eqns. 10 and 11). FDCD chromatograms were collected until good reproducibility was confirmed, which was at least three trials.

RESULTS AND DISCUSSION

Fluorescence and FDCD chromatograms

A mixture containing riboflavin and 4-methylumbelliferone was injected onto the chromatography system and the fluorescence was detected. The fluorescence chromatogram for one of these equivalent trials is shown in Fig. 3. Notice that the peak heights for both species are nearly identical. The FDCD detection system was then employed. The same sample mixture was injected onto the chromatography system with optimized FDCD detection. The FDCD chromatogram, for one of three trials, is shown in Fig. 4.

It is apparent that the optically inactive 4-methylumbelliferone still provides a signal in FDCD detection. This is due to the magnitude of its molar absorptivity, ε, and the magnitude of the ratio $\Delta I_0/I_0$, in relation to the magnitude of $\Delta\varepsilon$ for typical optically active species. Recall that the "error", R, due to ΔI_0 in a chromatography context for TMCD[10] is given by

$$R = \frac{\Delta I_0 \varepsilon}{I_0 \Delta\varepsilon} \tag{12}$$

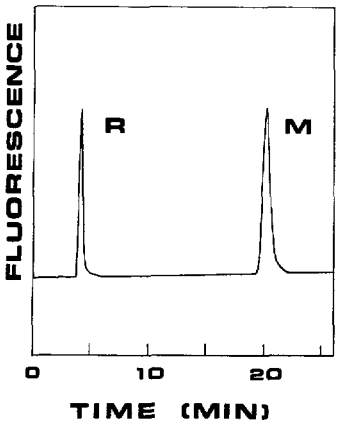

Fig. 3. Fluorescence chromatogram. R = (−)-riboflavin at $1.05 \cdot 10^{-5}$ *M*; M = 4-methylumbelliferone at $4.25 \cdot 10^{-6}$ *M*. 1 μl injected at these concentrations, at a flow-rate of 200 μl/min. Column, 5-μm ODS 150 × 2.1 mm; eluent, acetonitrile–water (20:80).

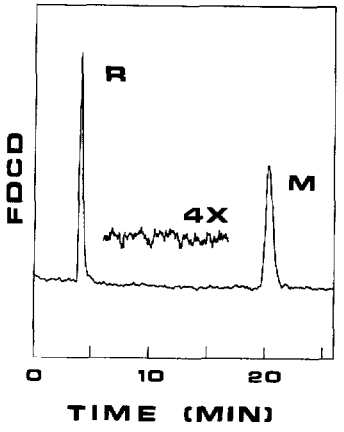

Fig. 4. FDCD chromatogram. R = (−)-riboflavin; M = 4-methylumbelliferone. Same chromatographic conditions and samples as in Fig. 3. A portion of the base line noise is amplified by a factor of 4 for comparison.

where R indicates the proportion of the offset signal as compared to the CD signal for optically active species.

Using the peak heights from the data in Figs. 3 and 4, and knowing the relative difference for the vertical scales used in Fig. 3 and 4, eqns. 10 and 11 were applied. For the optically inactive 4-methylumbelliferone, using eqn. 11, $\Delta I_0/I_0$ was found to be $5.5 \cdot 10^{-4}$. Using $\Delta I_0/I_0 = 5.5 \cdot 10^{-4}$ in eqn. 10, for optically active (−)-riboflavin, a $\Delta\varepsilon/\varepsilon$ of $4.9 \cdot 10^{-4}$ was calculated. Substituting these values into eqn. 12 yields an R value of 1.12. Thus, the fraction of the CD signal relative to the total signal measured in the FDCD experiment was calculated from the definition of R, and was found to be 47%. The $\Delta\varepsilon/\varepsilon$ value for (−)-riboflavin at 325 nm is generally near $5.0 \cdot 10^{-4}$, for solvent systems similar to the chromatographic eluent used in this work[8]. This value for $\Delta\varepsilon/\varepsilon$ compares favorably to the experimentally measured value of $4.9 \cdot 10^{-4}$ found here. Note that the $\Delta I_0/I_0$ contribution in eqn. 10 is added to the CD contribution, $\Delta\varepsilon/\varepsilon$. On different days, due to differences in alignment, the $\Delta I_0/I_0$ contribution was observed to either add or substract from the CD contribution. The direction of the CD contribution to the total FDCD signal for (−)-riboflavin is however consistent with eqn. 10, the literature $\Delta\varepsilon$ value, and the production of RCPL and LCPL via the modulation system.

Comparison of laser–modulation system combinations

It is interesting to compare the $\Delta I_0/I_0$ value obtained with FDCD–HPLC using a HeCd laser, with that obtained using an argon ion laser. Apparently, due to polarization instabilities in the HeCd laser and Pockels cell modulation system, a $\Delta I_0/I_0$ of $5 \cdot 10^{-4}$ and a peak-to-peak noise to signal ratio (PPN/I_0) of $7 \cdot 10^{-5}$ was the best obtainable. This was obtained at 150 kHz modulation frequency. Increasing or decreasing the modulation frequency had an adverse effect on these values. This is for either transmission or fluorescence detected CD measurements. In contrast, measurements with transmission and fluorescence detected CD systems using an argon ion laser[10] yielded a $\Delta I_0/I_0$ of $6 \cdot 10^{-5}$ and $1 \cdot 10^{-4}$, respectively. Also, the PPN/I_0 was near $1 \cdot 10^{-6}$ for the transmission detected CD system with a 500 kHz modulation frequency[10]. Obviously, there are marked differences in the characteristics of these two laser–modulation system combinations. We observed that transmission of the Pockels cell used is 90% at 488 nm and 75% at 325 nm. The absorption-induced heating at the latter wavelength probably caused the poorer performance. While the argon ion laser appears superior in considering $\Delta I_0/I_0$ and PPN/I_0, the HeCd laser was chosen since $\Delta\varepsilon/\varepsilon$ for riboflavin and other important biologically related fluorophores is more favorable at 325 nm (HeCd laser) than at 488 nm (argon ion laser).

Comparison of transmission and fluorescence detected CD

Comparing the nature of transmission and fluorescence detected CD measurements in the context of chromatography is quite useful. In the TMCD–HPLC system[10], the offset ratio, $\Delta I_0/I_0$, is readily observed on the lock-in amplifier as a steady DC signal shifted one way or the other from true zero. This is the chromatographic baseline. The elution of a highly absorbing species, assuming a large $\Delta I_0/I_0$, will cause the lock-in signal to swing back towards true zero, while a CD active species adds a contribution (hopefully significant) to this signal. In comparison, in the FDCD–HPLC system, the offset ratio, $\Delta I_0/I_0$, is not readily monitored since the correct

choice of filters will produce nearly a zero light background at the photomultiplier tube at the chromatographic baseline. It is necessary in FDCD–HPLC to consider the use of a steady-state flow of some highly fluorescing, yet optically inactive, species from another pumping system separate from the chromatography system. Thus, before a set of analyses are done on the FDCD–HPLC system, the offset ratio $\Delta I_0/I_0$ can be minimized with easy visualization on the lock-in amplifier. Once accomplished, the chromatography column can be connected to the detection cell to complete the FDCD–HPLC system. It should be emphasized that such careful adjustments to reduce $\Delta I_0/I_0$ are necessary to avoid artifacts in the chromatograms.

Quantitation calculations for the FDCD–HPLC system

It is of value to calculate the limit of detection (LOD) for (−)-riboflavin in the FDCD–HPLC chromatogram (Fig. 4). Using the 1 × PPN as the LOD, and the CD fraction (47%) of the peak height, the mass detectability is 168 pg injected (−)-riboflavin. The minimum concentration detectable is $4.5 \cdot 10^{-7}$ *M* injected, using the 1-μl injection loop, 1.2 cm observed pathlength cell, and a 10 s time constant. A 10-s time constant was used because the FDCD measurement is, actually, the difference of two very large numbers, with respect to the chromatographic background, for an eluting fluorophore. The peak widths here are not substantially degraded at a 10-s time constant. A 1-s time constant was tested, and for this FDCD–HPLC system, provided slightly sharper chromatographic peaks but a poorer LOD by a factor of about 4, as compared to the 10-s time constant data. The precision for multiple trials with the FDCD–HPLC system yielded a relative standard deviation of about 11% for the peak heights shown in Fig. 4. This indicates the presence of underlying polarization fluctuations with the measurement, that are hidden by the apparently stable chromatographic baseline. As a regular fluorescence detector (Fig. 3), the detection limit for riboflavin was about 300 femtograms, with a 10-s time constant.

CONCLUSIONS

A FDCD detection system, suitable for HPLC has been presented. The performance in terms of concentration detectability is better than that obtained for stopped-flow (static) or rapid-scanning CD detection systems[15,16]. The ability to do CD measurements in dynamic systems (HPLC) complements these published studies. The FDCD–HPLC system reported here provides optical activity identification of eluting species that would not have been available in the wavelength region used, unless microgram quantities of optically active materials were injected[17]. Thus, the system provides over a 10^3-fold improvement in mass detectability for fluorophores. The detectability is about 10 times better than the laser-based TMCD–HPLC system reported earlier[10]. With further refinements in laser sources and modulation systems[18], the selectivity of the measurement may be improved in the future. The present system provides the lowest detection limit available for indicating the optical activity of chromatographically separated species. Extension of this principle for microbore HPLC may improve this result, assuming a long enough pathlength can be maintained in detection without inducing too much band broadening. The principle of FDCD–HPLC may prove invaluable in the analysis of biologically significant samples, where the optical activity of the sample components must be known.

ACKNOWLEDGEMENTS

R.E.S. thanks the Phillips Petroleum Company for a fellowship. The Ames Laboratory is operated by the U.S. Department of Energy by Iowa State University under Contract No. W-7405-eng-82. This work was supported by the Office of Basic Energy Sciences.

REFERENCES

1 J. L. DiCesare and L. S. Ettre, *J. Chromatogr.*, 251 (1982) 1.
2 J. C. Kuo and E. S. Yeung, *J. Chromatogr.*, 223 (1981) 321.
3 J. C. Kuo and E. S. Yeung, *J. Chromatogr.*, 229 (1982) 293.
4 A. E. Watada and T. T. Tran, *J. Liq. Chromatogr.*, 8 (1985) 1651.
5 F. L. Vandemark and G. J. Schmidt, *J. Liq. Chromatogr.*, 4 (1981) 1157.
6 C. Y. W. Ang and F. A. Moseley, *J. Agric. Food Chem.*, 28 (1980) 483.
7 N. Ishibashi, T. Ogawa, T. Imasaka and M. Kunitake, *Anal. Chem.*, 51 (1979) 2096.
8 G. Tollin, *Biochemistry*, 7 (1968) 1720.
9 D. H. Turner, I. Tinoco, Jr. and M. F. Maestre, *Biochemistry*, 14 (1975) 3794.
10 R. E. Synovec and E. S. Yeung, *Anal. Chem.*, 57 (1985) 2606.
11 I. Tinoco, Jr. and D. H. Turner, *J. Am. Chem. Soc.*, 98 (1976) 6453.
12 H. P. Jensen, J. A. Schellman and T. Troxell, *Appl. Spectrosc.*, 32 (1978) 192.
13 E. W. Lobenstine and D. H. Turner, *J. Am. Chem. Soc.*, 102 (1980) 7787.
14 M. Young, in D. L. MacAdam (Editor), *Optics and Lasers* (*Springer Series in Optical Sciences*, Vol. 5), Springer, Berlin, Heidelberg, 1977, p. 188.
15 M. Anson and P. M. Bayley, *J. Phys. E*, 7 (1974) 481.
16 M. Hatano, T. Nozawa, T. Murakami, T. Yamamoto, M. Shigehisa, S. Kimura, T. Kakakuwa, N. Sakayanagi, T. Yano and A. Watanabe, *Rev. Sci. Instrum.*, 52 (1981) 1311.
17 S. A. Westwood, D. E. Games and L. Sheen, *J. Chromatogr.*, 204 (1981) 103.
18 K. W. Hipps and G. A. Crosby, *J. Phys. Chem.*, 83 (1979) 555.